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(11) CA 2 277 406 (13) A1

(43) 10.01.2000

(12)

(21) 2 277 406

(22) 08.07.1999

(51) Int. Cl.[6]: **C07K 014/76**, C07K 001/00, C07K 001/107, C07K 014/435, C07K 014/745

(30) 198 31 061.7 DE 10.07.1998

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(54) OBTENTION DE PREPARATIONS A BASE DE PROTEINES A TENEUR EN AGGREGATS REDUITE
(54) PRODUCTION OF PROTEIN PREPARATIONS WITH A REDUCED AGGREGATE CONTENT

(57)

The invention concerns a process for the production of protein preparations with a reduced aggregate content and in particular for the production of medical preparations of blood plasma proteins such as albumin.

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(12) (19) (CA) **Demande-Application**

(21) (A1) **2,277,406**
(22) 1999/07/08
(43) 2000/01/10

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(51) Int.Cl.[6] C07K 14/76, C07K 14/745, C07K 14/435, C07K 1/107, C07K 1/00
(30) 1998/07/10 (198 31 061.7) DE

(54) **OBTENTION DE PREPARATIONS A BASE DE PROTEINES A TENEUR EN AGGREGATS REDUITE**

(54) **PRODUCTION OF PROTEIN PREPARATIONS WITH A REDUCED AGGREGATE CONTENT**

(57) The invention concerns a process for the production of protein preparations with a reduced aggregate content and in particular for the production of medical preparations of blood plasma proteins such as albumin.

Abstract

The invention concerns a process for the production of protein preparations with a reduced aggregate content and in particular for the production of medical preparations of blood plasma proteins such as albumin.

Production of protein preparations with a reduced aggregate content

Description

The invention concerns a process for the production of protein preparations with a reduced aggregate content and in particular for the production of medical preparations of blood plasma proteins such as albumin.

Human plasma proteins have been purified on a large-scale for several decades and have been available for therapy and prophylaxis. Various methods can be used to prepare pure proteins or protein fractions from a complex plasma mixture such as fractionation by means of selective precipitation or separation of the protein mixtures by means of chromatographic methods such as ion exchange chromatography, gel filtration and affinity chromatography. These methods are also very often combined in order to obtain optimal results.

Precipitation methods with ethanol have also been successfully used for a long time to fractionate plasma proteins on a large-scale (Cohn et al., J. Am. Chem. Soc. 68 (1946), 459-475; Kistler and Nitschmann, Vox Sang. 7 (1962), 414-424). This method is still used nowadays worldwide to isolate large amounts of plasma proteins, in particular albumin, immunoglobulins and coagulation factors and also other proteins from human plasma especially for medical applications. However, ethanol fractionation of plasma proteins has some disadvantages. Thus a partial denaturation of sensitive proteins can occur especially at high alcohol

concentrations or/and high temperatures. Such a denaturation can lead to the partial or complete loss of the physiological function or to structural changes which can manifest themselves as activation of proenzymes or formation of new antigen determinants or protein aggregates.

In order to inactivate potential infectious contaminants e.g. viruses or other pathogens, it is possible to subject blood plasma preparations to a final pasteurisation step. Albumin can for example be pasteurised by heating to 60 to 64°C for 10 h in the presence of stabilizers such as N-acetyl tryptophanate or sodium caprylate (Gellis et al., J. Clin. Invest. 27 (1948), 239-244). Other pasteurisation methods are disclosed for example in the US patents 2,897,123; 3,227,626; 4,379,085; 4,440,679; 4,623,717 and 4,803,073. Pasteurised preparations of plasma proteins have proven to be very safe with regard to the transmission of viruses and pathogens. An additional advantage of pasteurisation is that it is not necessary to add toxic additives to the protein preparation and thus in many cases an inactivation of pathogens is possible in the final container. However, a disadvantage of pasteurisation is that there is often a considerable increase of aggregate formation.

However, this aggregate formation which leads to the formation of visual or subvisual particles sometimes in high amounts, is extremely undesirable especially for medical and pharmaceutical applications. Thus large particles can directly impair the function of capillary vessels. Undesired side-effects of subvisual particles or protein aggregates are known and have been described for the various protein formulations. Hence, most of the

approval authorities insist on limits for the aggregate content of for example albumin or immunoglobulin solutions. Aggregates in immunoglobulin solutions can for example trigger an uncontrolled activation of the complement system and lead to serious side-effects. It has been described that albumin aggregates disappear very rapidly from the circulation, probably block the RES system and sensitize the organism for states of shock. Hence aggregates in protein solutions can lead in extreme cases to life-threatening situations which have to be avoided under all circumstances.

Due to the very broad range of applications of plasma proteins there is a major need for processes which can improve the safety, tolerance and biological activity of the proteins. In addition such methods should be economic and simple to use.

According to the present invention it was surprisingly found that protein preparations with a reduced aggregate content can be produced by improving a thermal treatment for example to eliminate potential infectious contaminants, by a prior or subsequent separation step such that denaturation or/and aggregate formation is significantly reduced or prevented in the final product. The desired active and native ingredient is enriched by the separation step in a protein or protein mixture which is subsequently to be thermally treated. Hence a subject matter of the present invention is a process for the production of protein preparations with a reduced aggregate content comprising a thermal treatment and the process is characterized in that aggregates, denatured proteins or/and contaminants present in the protein preparation are separated before or/and after the thermal treatment.

The process according to the invention is particularly suitable for the production of preparations of blood plasma proteins but is not limited thereto. The blood plasma proteins are preferably selected from the group comprising plasminogen, albumin, factor VIII, factor IX, fibronectin, immunoglobulins, kininogen, antithrombin III, α-1-antitrypsin, pre-kallikrein, fibrinogen, thrombin, (apo)-lipoproteins and blood plasma protein fractions which contain one or several of these proteins. It is particularly preferred to produce a preparation of albumin, immunoglobulins or (apo)-lipo-proteins and most preferably an albumin preparation.

An albumin preparation with a reduced aggregate content produced by the process according to the invention is considerably more suitable for medical applications than preparations of the prior art. The albumin preparations according to the invention can be used as plasma expanders and also to treat burns. In addition other applications are also possible such as ultrasound imaging or as a stabilizing additive to protein solutions especially if they are highly active proteins or peptides at low concentrations such as hormones, chemokines, cytokines or enzymes which nowadays are often produced using recombinant technology.

The process according to the invention is also particularly suitable for the production of preparations of chemically modified proteins in which case the functional groups of the proteins, in particular functional side chains, are modified. Chemically modified proteins, e.g. albumin, can for example be used as carriers of functional molecules which in vivo do not reach the desired compartments of the organism or which do not have the desired activity in a free form. The

chemical modification is preferably selected from the group comprising polyethylene glycol modification, iodination, acylation e.g. acetylation, oxidation e.g. with peroxides, nitroxylation and cross-linking e.g. with bifunctional linkers.

The essential step in the process according to the invention i.e. an at least partial separation of aggregates, denatured proteins or/and contaminants before or/and after a thermal treatment can comprise a precipitation e.g. with ammonium sulfate, ethanol or polyethylene glycol combined with a separation of the precipitated aggregates by known methods e.g. by centrifugation. In addition the separation can also include a gel filtration e.g. with Fractogel EMD Bio SEC (Merck) or with other gel filtration media such as Sephadex or Sepharose (Pharmacia). However, the separation is preferably carried out by membrane filtration using membranes of a suitable pore size e.g. with an exclusion size of 30 to 1000 kD and in particular of 100 to 500 kD which separates undesired components with a molecular weight above the exclusion size. The separation can also additionally include a nanofiltration such as that which is used to remove viruses e.g. using the filtration materials DV20 or DV50 (Pall Corp., New York, USA) or Planova 15N (Asaki, Tokyo, JP).

The thermal treatment which is carried out before or preferably after the separation preferably comprises a temperature increase for a long period e.g. a pasteurisation step which can be carried out in a known manner. The thermal treatment usually comprises heating the protein solution to at least 55°C for an adequate period in order to at least substantially inactivate

infectious contaminants. The duration of the heating is preferably at least 5 h, particularly preferably ca. 10 h. The temperature during the heating is preferably in a range between 60 and 65°C. In order to prevent inactivation of the proteins, the thermal treatment is preferably carried out in the presence of known stabilizers such as N-acetyl tryptophanate or sodium caprylate in the case of albumin.

Whereas the aforementioned separation step already adequately reduces the formation of aggregates, denatured proteins or/and contaminants for many protein mixtures, in other cases the desired results can only be obtained by carrying out a pretreatment step before the separation in which components in the protein preparation that can aggregate or/and denature are converted into a state that can be separated. The pretreatment step preferably comprises changing the physicochemical parameters in the protein preparation, in particular a stress treatment in order to aggregate denatured, partially denatured or sensitive molecules or contaminants that are already present in the preparation or to convert them otherwise into a state which can be removed in the subsequent separation step. This change of physicochemical parameters can for example be a change of the pH, a change and in particular an elevation of the temperature, a change in the ionic strength or the dielectric constant, the application of shear forces or a combination of two or several of these measures.

This pretreatment step particularly preferably comprises an elevation of the temperature of the protein preparation. The extent and duration of this temperature increase depend on the respective protein preparation or

its sensitivity. On the one hand the temperature increase and its duration should be sufficient to convert the largest possible proportion of aggregatable components into a state which can be separated; on the other hand the selected conditions should be not too drastic in order to largely avoid inactivation of the desired proteins in the preparation. It has proven to be advantageous for many proteins such as albumin to heat the protein preparation for a period of 30 min to 4 h, in particular for 1 h to 2.5 h to a temperature of 40 to 70°C, in particular of 45 to 65°C.

The process according to the invention is particularly suitable for the production of medical protein preparations with a reduced aggregate content. Compared to an otherwise identical protein preparation without the separation step, the aggregate content is preferably reduced by at least 10 %, particularly preferably by at least 30 % and most preferably by at least 50 %. In some cases the aggregate content is even reduced by 90 % or more.

Finally the invention also concerns a protein preparation with a reduced aggregate content which has been produced by the process according to the invention. This protein preparation is considerably more suitable especially for medical applications than preparations of the prior art since it causes considerably fewer intolerance reactions when used therapeutically.

Finally the invention is also elucidated by the following examples.

Examples

Example 1

An albumin solution (10 % in 10 mmol/l NaCl) was incubated for 90 min at 45°C. Subsequently the solution was diafiltered through a cassette with a molecular weight cut off of 300 kD. The ultrafiltrate which mainly contains monomeric albumin was pasteurised for 10 h at 60°C in the presence of stabilizers (16 mmol/l Na caprylate, 16 mmol/l acetyl tryptophan).

Results:

The aggregate content was 2 % after the preincubation, > 0.1 % in the ultrafiltrate after the ultrafiltration and 0.6 % after pasteurisation. A strong accumulation of aggregates was measured (> 80 %) in the retentate of the ultrafiltration. An aggregate content of 6 % was measured after the pasteurisation without preincubation and subsequent diafiltration. The results were not significantly changed by adding stabilizers (N-acetyl tryptophanate and sodium caprylate) during the preincubation of the albumin.

An analogous experiment was carried out with a commercially available albumin solution (20 % in 140 mmol/l NaCl, 12 mmol/l N-acetyltryptophanate and Na caprylate). The behaviour of the solutions during the experiment and the results were essentially identical to those of the 10 % albumin solution as a starting material.

Determination of the aggregate content in solutions:

1 mg protein was separated by means of high performance gel filtration on a Superose HR 10/30 column (Pharmacia) in 70 mmol/l potassium phosphate buffer, pH 7.0 at a flow of 1.0 ml/min. The detection was carried out by determining the absorbance at 280 nm. The monomer and aggregate content of the samples was calculated from the areas of the protein peaks.

Example 2

An albumin solution (10 % containing stabilizers 8 mmol/l in each case) was adjusted to pH 10.5 with 1 mol/l NaOH and subsequently heated for 2 h at 65°C. Afterwards the solution was cooled to room temperature and the pH value was back titrated to 7 with 1 mol/l HCl.

The aggregates that had formed were removed by selective precipitation as follows:

(a) Ammonium sulfate was added to the solution up to a saturation of 25 %, 30 % and 35 %. The turbidity which subsequently occurred was removed by centrifugation and the supernatant was re-equilibrated by means of gel filtration on Sephadex G-25 (PD-10, Pharmacia) in 150 mmol/l NaCl. Subsequently it was pasteurised for 10 h at 60°C in the presence of a stabilizer (Na caprylate, N-acetyltryptophanate, 8 mmol/l in each case).

(b) Polyethylene glycol (PEG) 3000 was added to the solution up to a final concentration of 6.1 %, 7.1 %

and 8.3 % and the turbidity which this caused was removed by means of centrifugation. The supernatant was subsequently re-equilibrated as described in (a) and pasteurised in the presence of stabilizers to obtain the final product.

Results:

		% aggregate content in the final product	% Decrease of aggregate formation relative to a control
Albumin without pretreatment (control)		22.9	
Ammonium sulfate	25 %	15.8	31
	30 %	3.7	84
	35 %	2.8	88
PEG	6.1 %	20.5	10
	7.1 %	16.8	27
	8.3 %	9.3	59

Example 3

Activation of PEG:

5.5 g cyanuric chloride was dissolved in 400 ml anhydrous benzene containing 10 g sodium carbonate. 19 g PEG 1900 was added to the mixture and it was stirred overnight at room temperature. The solution was filtered and 600 ml petroleum ether was added slowly. The suspension was filtered and the precipitate was dissolved in 400 ml benzene. The precipitation and filtration process was repeated several times in order to remove free cyanuric chloride.

Binding of the activated PEG to albumin:

1 g albumin was dissolved in 100 ml 0.1 mol/l sodium tetraborate, pH 9.2. 8 g activated PEG was added at 4°C and the pH was kept at 9.2 for one hour. In this manner 80 to 90 % of the primary amino groups were modified with PEG. After the reaction, excess PEG was removed by diafiltration (10 kD cut off membrane) and the solution was rebuffered against 10 mmol/l NaCl. The solution was subsequently preincubated at 45°C as described in example 1, diafiltered through a 300 kD cassette and pasteurised for 10 h at 60°C in the presence of stabilizers.

Results:

An aggregate content of 10 % was measured in PEG-modified albumin without preincubation. It was possible to reduce the aggregate content to 4.5 % by preincubation and subsequent diafiltration.

Example 4

A 20 % albumin solution was diluted with 0.1 mol/l borate buffer pH 9.5 to 10 % and cooled to 0°C. Subsequently 20 % (v/v) cold KI_3 solution was added and it was incubated for 30 min at 0°C. The reaction was stopped by addition of a few drops of $NaSO_3$ (1 mol/l), the protein solution was incubated for 2 h at 60°C and cooled. An aliquot was incubated for 8 h at 60°C (to assess the aggregate formation).

The remainder of the solution was diafiltered over a 300 kD membrane against 4.5 volumes of 140 mmol/l NaCl. Finally the retentate was concentrated to a protein concentration of 20 %. The ultrafiltrate was rebuffered by means of 10 kD diafiltration: diafiltration against 10 volumes 140 mmol/l NaCl and subsequent concentration on the 10 kDa diafiltration membrane to a protein content of 15 to 20 %. The resulting protein solution was pasteurised as in example 1 in the presence of stabilizers.

An aggregate content of 5.2 % was achieved in the iodated albumin with pretreatment (preincubation, 300 kD diafiltration); an aggregate content of 12.7 % was measured without pretreatment i.e. a reduction of 59 % was achieved.

Example 5

Albumin (20 %) was diluted 1:2 with saturated Na acetate solution pH 7.5 and cooled to 0°C. Acetic anhydride was metered in portions within 1 h (same weight as the added protein). Subsequently the pH was adjusted to 7.5, the sample was filtered through a 1.2 µm filter and rebuffered by means of 10 kD diafiltration (against 30 volumes 140 mmol/l NaCl).

The protein solution was incubated for 2 h at 60°C, then cooled and diafiltered over a 300 kD membrane against 4 volumes 140 mmol/l NaCl. Finally the retentate was concentrated to ca. 20 %. The ultrafiltrate was rebuffered by means of 10 kD diafiltration (diafiltration against 10 volumes 150 mmol/l NaCl) and concentrated on the same membrane to a protein content

of 15 - 20 %. The solution of acetylated albumin was subsequently pasteurised in the presence of stabilizers as described in example 1.

An aggregate content of 62 % was measured in the final product with pretreatment (preincubation, 300 kD, diafiltration); the acetylated product gelled without the pretreatment i.e. soluble protein was no longer present.

Example 6

An albumin solution (10 %) containing 0.5 mmol/l EDTA was adjusted to pH 3.2 with 0.1 M perchloric acid and heated to 30°C. H_2O_2 was added to the protein solution to a final concentration of 0.5 mmol/l. Subsequently it was incubated for 2 h at 30°C. After the incubation a 10 kD diafiltration was carried out against 4 volumes 140 mmol/l NaCl. The retentate was diafiltered over a 300 kD membrane against 2 volumes 140 mmol/l NaCl, and finally the retentate was concentrated to a protein concentration of 15 to 20 %.

The ultrafiltrate was rebuffered by means of 10 kD diafiltration (10 volumes 140 mmol/l NaCl) and concentrated on the same membrane to a content of 10 to 15 % protein. The solution of oxidized albumin was subsequently pasteurised in the presence of stabilizers as described in example 1.

An aggregate content of 1.1 % was measured in the final product with pretreatment (300 kD diafiltration). Without pretreatment the oxidized albumin had an aggregate content of 11.1 %. The aggregate content could

hence be reduced by 90 %.

Example 7

An albumin solution (10 %, 20 ml) was adjusted to pH 8.5 to 9.0 with NaOH. 50 mg dimethyl suberimidate suspended in 2 ml triethanolamine at pH 9.7 was added to this and it was incubated for 2 h at room temperature. The reaction was stopped by addition of 10 % (v/v) 0.1 mol/l Tris pH 8.5. The protein solution was subsequently incubated for 2 h at 60°C and then cooled. 15 ml of this solution was diafiltered over a 300 kD membrane (against 12 volumes 140 mmol/l NaCl). Finally the retentate was concentrated as strongly as possible.

The ultrafiltrate was rebuffered by means of 10 kD diafiltration against 30 volumes 140 mmol/l NaCl and subsequently concentrated to a content of 15 to 20 % protein by means of vacuum dialysis against 140 mmol/l NaCl. The solution of albumin modified by the cross linker was subsequently pasteurised in the presence of stabilizers as described in example 1.

An aggregate content of 2.5 % was measured in the final product with pretreatment (300 kD diafiltration). Without pretreatment the modified albumin had an aggregate content of 20.5 %. The aggregate content could hence be reduced by 88 %.

Example 8

4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidin-1-oxyl (BrAcTPO; dissolved in ethanol, 500 mg/ml) was added to

an albumin solution (10 % protein, in 10 % ethanol) (0.177 g BrAcTPO/g protein). The solution was heated to 45°C, the pH was adjusted to 9.5 with NaOH and kept at this pH for 2 to 3 h by the continuous addition of lye. Subsequently the pH was back titrated to 7.2 with HCl and the solution was heated for 90 min to 60°C. Afterwards a 300 kD diafiltration with 15 volumes 140 mmol/l NaCl was carried out. The ultrafiltrate was diafiltered with a 10 kD membrane, 5 to 10 volumes 140 mmol/l NaCl and finally concentrated on the same membrane to a protein content of 20 %. The solution was pasteurised in the presence of stabilizers as described in example 1.

An aggregate content of 0.1 % was measured in the polynitroxylated albumin with pretreatment (incubation, 300 kD diafiltration). Without pretreatment the modified albumin had an aggregate content of 5.5 %. The aggregate content could hence be reduced by 90 %.

Example 9

A solution of apolipoprotein A-I (10 g/l in 10 mmol/l NaCl) was incubated for 2 h at pH 5.0 and 60°C. Subsequently the pH was adjusted to 7.5 and guanidine HCl was added to a concentration of 2 mol/l and incubated for 2 h at 45°C. This solution was diafiltered over a 300 kD membrane (10 vol 10 mmol/l NaCl) and subsequently diafiltered with a 10 kD membrane and concentrated to 10 g/ml.

Aggregate contents of 2.4 % to 5.4 % were measured in the apolipoprotein A-I solutions without pretreatment (4 experiments); the aggregate content was reduced to 0.4 to 0.8 % with pretreatment (3 experiments).

Example 10

A solution of immunoglobulin G (20 g/l, ≤ 3 mmol NaCl/l) was incubated for 12 h at pH 7.0 and 45°C. The content of aggregates increased during the pretreatment from < 0.1 to 1.0 %. It was subsequently gel filtered over an acrylic gel (Sepharyl S-300 HR), the pH was adjusted to 5.3 with HCl and it was concentrated with a 10 kD membrane to 120 g/l. The aggregate content was 0.2 % and remained stable during storage for 6 months at 37°C. The content increased to 0.8 % under the same conditions in a non-treated reference solution.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. Process for the production of protein preparations with a reduced aggregate content including a thermal treatment,
wherein
aggregates, denatured proteins or/and contaminants present in the protein preparation are separated before or/and after the thermal treatment.

2. Process as claimed in claim 1,
wherein
a preparation of blood plasma proteins is produced.

3. Process as claimed in claims 1 or 2,
wherein
the blood plasma proteins are selected from the group comprising plasminogen, albumin, factor VIII, factor IX, fibronectin, immunoglobulins, kininogen, antithrombin III, α-1-antitrypsin, pre-kallikrein, fibrinogen, thrombin, (apo)-lipoproteins and blood plasma protein fractions which contain one or several of these proteins.

4. Process as claimed in one of the claims 1 to 3,
wherein
a preparation of albumin, immunoglobulin or (apo)lipoproteins is produced.

5. Process as claimed in one of the claims 1 to 4, **wherein** a preparation of chemically modified proteins is produced.

6. Process as claimed in claim 5, **wherein** the chemical modification is selected from the group comprising polyethylene glycol modification, iodination, acylation, oxidation, nitroxylation and cross-linking.

7. Process as claimed in one of the claims 1 to 6, **wherein** the thermal treatment comprises heating the protein preparation to at least 55°C for an adequate period in order to at least substantially remove infectious contaminants.

8. Process as claimed in one of the claims 1 to 7, **wherein** the duration of the thermal treatment is at least 5 h.

9. Process as claimed in one of the claims 1 to 8, **wherein** the thermal treatment is carried out in the presence of stabilizers.

10. Process as claimed in one of the claims 1 to 9, **wherein** the separation includes a precipitation step.

11. Process as claimed in claim 10,
wherein
the precipitation is carried out by adding ammonium sulfate, ethanol or polyethylene glycol.

12. Process as claimed in one of the claims 1 to 11,
wherein
the separation includes a gel filtration step.

13. Process as claimed in one of the claims 1 to 12,
wherein
the separation includes a nanofiltration.

14. Process as claimed in one of the claims 1 to 13,
wherein
the separation includes a membrane filtration.

15. Process as claimed in claim 14,
wherein
an exclusion size of 30 to 1000 kD is selected for the membrane filtration.

16. Process as claimed in one of the previous claims,
wherein
a pretreatment step is carried out before the separation in which components present in the protein preparation which can aggregate or/and be denatured are converted into a state that can be separated.

17. Process as claimed in claim 16,
wherein
the pretreatment step comprises changing the physicochemical parameters in the protein preparation.

18. Process as claimed in claim 17,
wherein
the change in physicochemical parameters comprises a change of the pH, an increase of the temperature, a change of the ionic strength or of the dielectic constant, the application of shear forces or a combination of two or several of these measures.

19. Process as claimed in claim 18,
wherein
the temperature of the protein preparation is increased.

20. Process as claimed in claim 19,
wherein
the protein preparation is heated for 30 min to 4 h to a temperature of 40°C to 70°C.

21. Process as claimed in claim 20,
wherein
the protein preparation is heated for 1 h to 2.5 h to a temperature of 45 - 65°C.

22. Use of the process as claimed in one of the claims 1 to 21 to produce medical protein preparations with a reduced aggregate content.

23. Use as claimed in claim 22,
wherein
the aggregate content is reduced by at least 10 % compared to a protein preparation without a separation step.

24. Use as claimed in claim 23,
wherein
the aggregate content is reduced by at least 50 %.

25. Protein preparation with a reduced aggregate content produced by a process as claimed in one of the claims 1 to 21.